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Morphological, phylogenetic and physiological diversity of cyanobacteria in the hot springs of Zerka Ma'in, Jordan

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Abstract

The freshwater thermal springs of Zerka Ma'in, located in Jordan in the mountains of Moab east of the Dead Sea, are densely inhabited by cyanobacteria up to the highest temperature of 63 °C. We have investigated the cyanobacterial diversity of these springs and their outflow channels by microscopic examination, culture-dependent and culture-independent phylogenetic analysis, and by physiological studies of selected isolates of special interest. Both unicellular and filamentous types of cyanobacteria are present, and we identified morphological types such as *Thermosynechococcus*, *Chroogloeocystis*, *Fischerella* (*Mastigocladus*), *Scytonema* (occurring as large masses at lower temperatures), and others. Although morphologically similar cyanobacteria have been identified in hot springs world-wide, the Zerka Ma'in strains were phylogenetically distinct based on 16S rRNA gene sequence analysis. Considerable diversity was detected also in the gene sequences of *nifH* (nitrogenase reductase), encoding one of the key enzymes involved in nitrogen fixation. Nitrogen fixation in a *Mastigocladus* isolate obtained from the springs was investigated in further depth. The heterocystous strain could fix nitrogen (as assayed by acetylene reduction) at temperatures up to 53 °C.

Keywords

Cyanobacteria, Jordan, Zerka Ma'in, thermophilic cyanobacteria, biodiversity, 16S rRNA phylogeny, nitrogen fixation

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Introduction

The hot springs (up to 63 °C) of Zerka Ma'in, located in Jordan in the mountains of Moab near the north-eastern end of the Dead Sea (Fig. 1) were first mentioned by the Jewish-Roman historiographer Flavius Josephus (37 to c. 100 C.E.): "In the ravine which encloses the town [Herod's fortress Machaerus; the present day ruins of Makaur] on the north, there is a place called Baaras. … In this same region flow hot springs, in taste widely differing from each other, some being bitter, while others have no lack of sweetness."

The first explorer to reach the Zerka Ma'in hot springs was the German Ulrich Jasper Seetzen (1767–1811), who visited the site just over 200 years ago. In the report of his visit to the site in 1807 he mentioned the presence of green slimy material that consists of microscopic algae: "These springs are about two hours distant from the Dead Sea, to which the track from here appears to be very difficult. In the water grew a green slimy small alga" (Seetzen 1854; translation A.O.). It is curious that none of the later explorers who visited the site in the course of the 19th century and the first years of the 20th century mentioned the so prominent green growth in the waters of the hot springs (Figs 2A-C). However, a few observations on the microbial mats in the springs were published by the German geologist Max Blanckenhorn, who surveyed the area in 1908: "An algologist could find here, as well as in the other hot sulfur springs of Palestine, a wonderful area for observations and collection. … There where the water was particularly hot, blue-green Cyanophyceae appeared to dominate. For the rest, the whole bottom of the stream and the rocks present in it are covered by green mats. These felt-like mats are often small in the form of a sponge or pillow with a dark-green, somewhat wrinkled skin …" (Blanckenhorn 1912; translation A.O.).

The cyanobacterial flora of the nearby hot springs of the Zara area near the shore of the Dead Sea, the site of the ancient Kallirrhoë (Donner 1963), was surveyed in 1936 (Frémy and Rayss 1938; Rayss 1944). However, no studies of the cyanobacteria of Zerka Ma'in have been conducted until 2005. The peace treaty between Jordan and

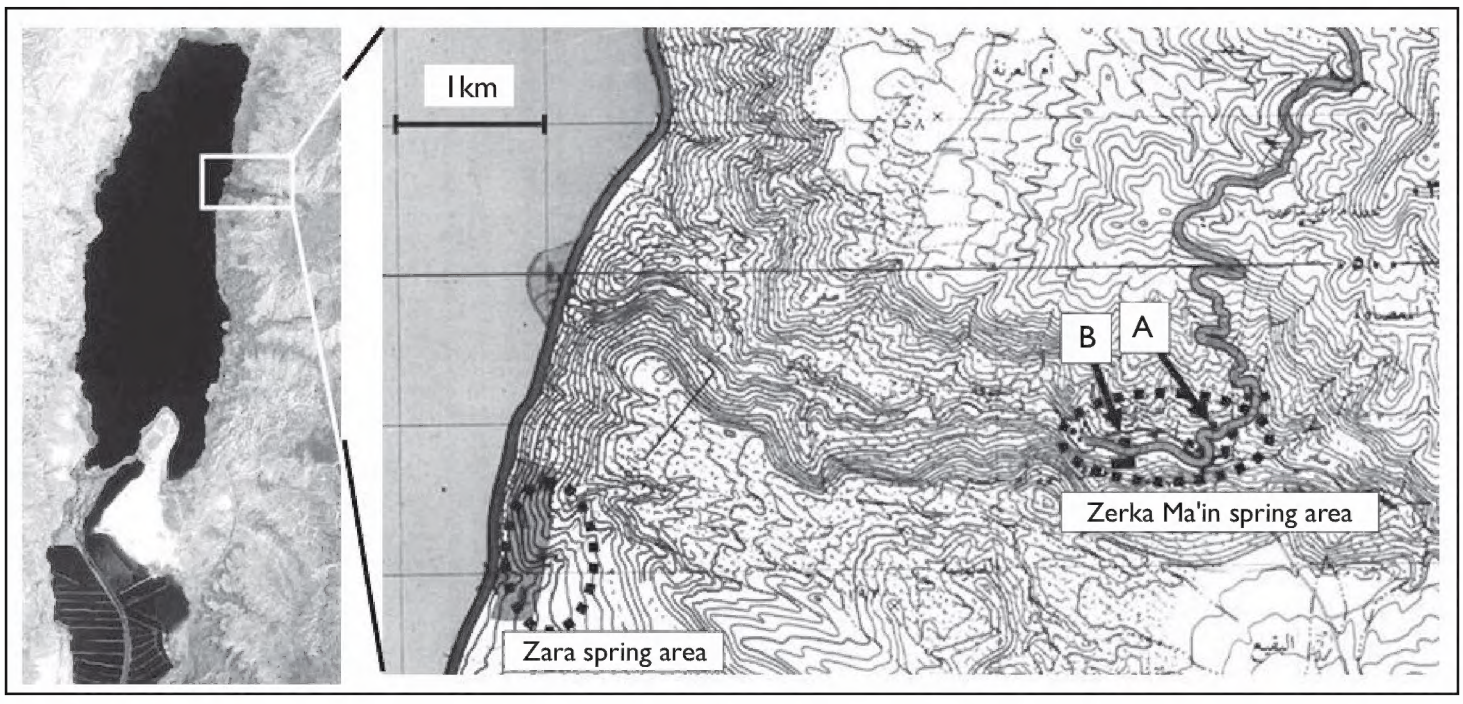

Figure 1. Satellite image of the Dead Sea and the location of the Zerka Ma'in spring area.

Israel of 1994, and the establishment of the Bridging the Rift Foundation in the year 2000, promoting peace in the Middle East through science, enabled us to perform the first biological surveys to characterize the highly interesting microbial communities of Zerka Ma'in and to discover some of its many interesting and sometimes unique features. Sponsored by the Bridging the Rift Foundation, our team of Jordanian and Israeli scientists has made a number of sampling trips to the site in 2005 to 2007 (Ionescu et al. 2007, 2009; Oren et al. 2008). We here present some of the results of our cyanobacterial diversity studies of the Zerka Ma'in hot springs, based both on microscopical characterization of the organisms present and on molecular, 16S rRNA gene-based techniques.

Materials and methods

Sample collection, cyanobacterial cultivation and identification

Cyanobacterial mats were collected from the Zerka Ma'in springs on December 14, 2005, November 16, 2006 and July 3, 2007. Samples were transferred to glass vials

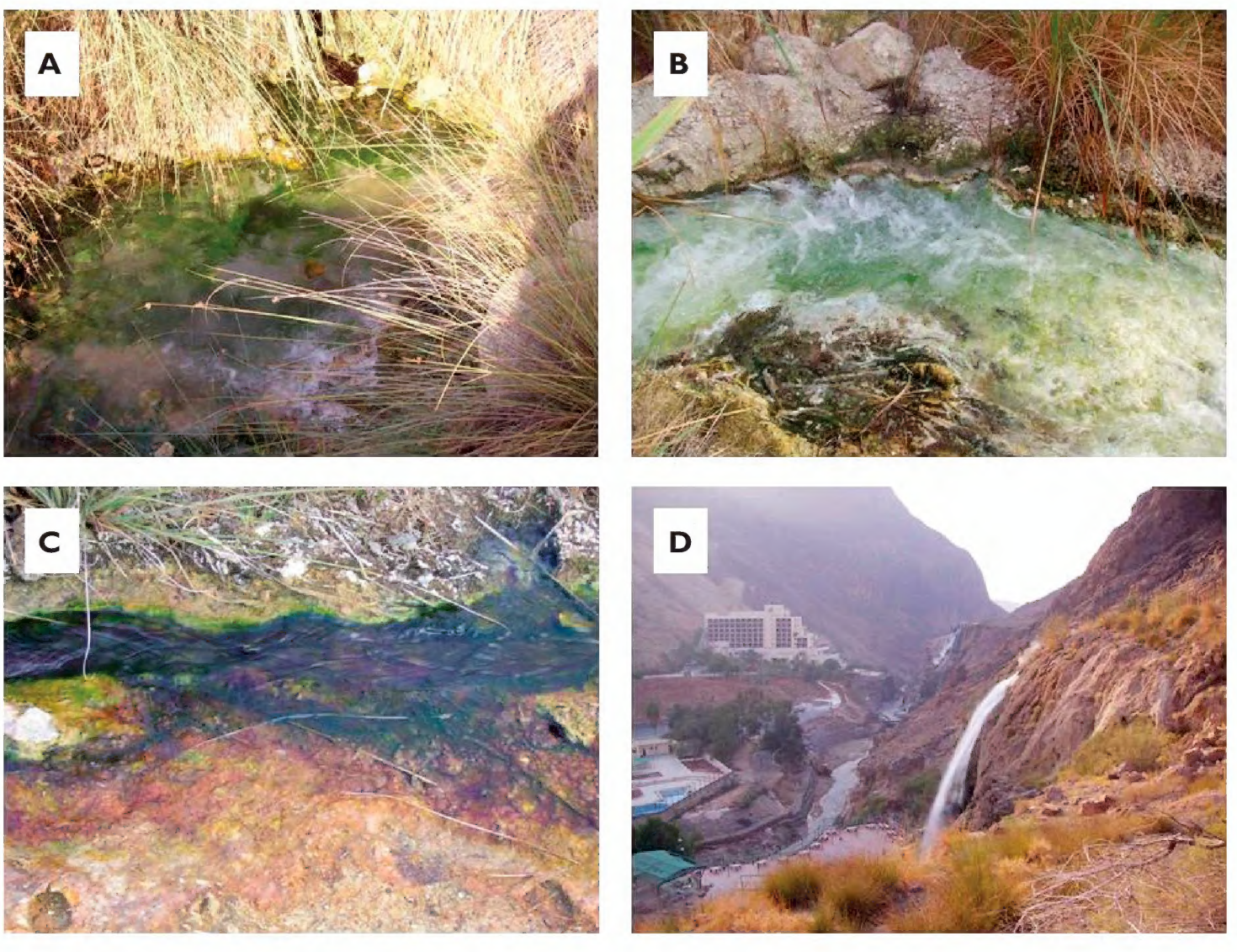

Figure 2. Views of the thermal spring area of Zerka Ma'in. Panel A shows one of the springs in Area A indicated in Fig. 1, Panel D provides an overview of the waterfalls with hot water of about 58 °C to 60 °C in Area B.

and tubes and used for microscopic identification of the organisms present, isolation of cyanobacterial strains, and molecular studies. Most experimental work referred to below was done based on the July 2007 samples. Site A (Figs 1, 2A) consists of two pools, the upper one flowing into a lower one through a pipe. The temperature of the upper one was 63 °C during all sampling trips. The lower pool ranged between 62 °C and 63 °C. The upper pool is shallow (ca 50 cm) and surrounded by rock walls. Green mats were found on the rocks, at and slightly above the water air interface. Submerged rocks are covered by mats as well. The lower pool is deeper, larger and surrounded by vegetation. Site B (Fig. 1B) is located ca 500 m west of site A. Green mats are found on the channels' earth banks and on submerged rocks. An orange mat is often found beneath the green mat. Site C (Fig. 1C) is a stream located 25 m above site A. The main stream is a combination of two smaller ones at a temperature of 25 °C and 51 °C. Over a distance of 25 m from the confluence point the temperature of the stream reaches 39 °C. The water from the springs at site B flows through a 50 m channel that ends in a waterfall (Fig. 1D). The temperature of the water was 59 °C along the entire channel.

Samples were examined and photographed in a Zeiss Axiovert 135 TV microscope equipped with phase-contrast optics. Morphological types were identified to the genus level on the basis of the identification systems proposed by Geitler (1932) and the "form-genus" approach of Castenholz (2001). When relevant, names in common use but without standing in the botanical and bacteriological nomenclature were used as well (e.g. *Thermosynechococcus*). We have isolated representative types of filamentous and unicellular cyanobacteria by enrichment and direct isolation on agar plates of growth medium BG-11, using incubation temperatures of 45 °C and 55 °C. Cultures are maintained on agar slides in the laboratory of A.O.; the filamentous *Mastigocladus*-like strain nBTRCC 101 has been submitted for deposition in the UTEX - The culture collection of algae at the University of Texas at Austin (temporary accession number: ZZ867).

Sequencing and analysis of cyanobacterial 16S rRNA genes

For molecular 16S rRNA-sequence based analysis, samples were placed in 15 ml sterile tubes containing 2 ml of lysis buffer (100 mM Tris-HCl, 50 mM EDTA, 10 mM NaCl, 1% SDS, pH 8), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1). After washing the extracts with chloroform-isoamyl alcohol (24:1), DNA was precipitated with cold ethanol, washed with ice-cold 70% ethanol, and resuspended in water. Fragments of the 16S rRNA gene were amplified by PCR, using cyanobacteria-specific primers as specified in Ionescu et al. (2009). The primer sets 29F – 809R and 740F – 1494R were used for cultures, while 106f and 781R (Nübel et al. 1997; Ionescu et al. 2009) were used for environmental samples. Amplicons originating from environmental sequences were cloned using the InsTAclone kit (K1214, Fermentas, Lithuania) and verified using colony PCR. Successful reactions

were sent for cloning and sequencing at the Genome Sequencing Center at Washington University, St. Louis, MO. To obtain reliable results each clone was sequenced in both directions. Each individual sequence used for the phylogenetic analysis is the result of two aligned sequences from the same clone. All sequences were compared to the NCBI nr databases using the NetBlast application (available from NCBI). The top five hits as well as some additional relevant sequences were used for phylogenetic analysis. Sequences were aligned using the Muscle 3.6 software (Edgar 2004). Phylogenetic trees were constructed using the MEGA 4.0 (Tamura et al. 2007). The Distance Matrix was calculated using the Jukes-Cantor algorithm and the trees were constructed using the Minimum Evolution method. Validity of tree topology was evaluated using the bootstrap method (100 replicates). Environmental 16S rRNA gene sequences from Zerka Ma'in are available from the GenBank at accession numbers EU326950-327016.

Nitrogen fixation studies

To assess the importance of nitrogen fixation to the cyanobacteria in the Zerka Ma'in springs, we used the acetylene reduction test to quantify the nitrogenase activity of the community. Biomass was collected from the major spring of Area A (63 °C) and from two nearby streams with temperatures of 51 °C and 39 °C. Acetylene reduction tests were performed *in situ* in the light and in the dark for 1:45–2:45 hours, incubation times being limited due to logistic constraints. Full details of the experimental conditions were given by Ionescu et al. (2009).

Results

Physical and chemical properties of the samples collected

The major springs that issue at sites A and B as indicated in Fig. 1 had a temperature of 62 °C to 63 °C, independent of the season of sampling. Most samples collected from the springs and their outflow channels had temperatures between 63 and 51 °C. During our last sampling trip (June 2007) we found a channel located about 100 m north of the major springs of site A, which had not been surveyed previously. Its water temperature was 39 °C.

The waters of the springs differed little in chemical properties. The total dissolved salts concentrations ranged between 1,267 and 1,445 mg/L, with an alkalinity of 110–130 mg/L. A typical analysis (water from site A pictured in Fig. 2A) gave (mg/L): Cl^-, 810; SO_4^{2-}, 196; Ca^{2+}, 186; Mg^{2+}, 91; Na^+, 86; K^+, 35. Up to 0.3 mM sulfide was measured in the water sampled near the sources. The pH ranged from 6.4 to 6.8. More detailed chemical analyses have been reported elsewhere (Abu Ajamieh 1980, 1989; Rimawi and Salameh 1988; Ionescu et al. 2009).

Microscopical observations of the spring samples and the cyanobacterial cultures obtained

Microscopical examination of samples collected from the springs and their outflow channels showed a dominance of unicellular cyanobacteria. Figure 3 presents a representative selection of organisms encountered at temperatures between 58 °C and 63 °C. At the highest temperatures the cyanobacterial mats were dark green in colour (Figs 2A, B). Here unicellular *Thermosynechococcus*-type cyanobacteria dominated (Fig. 3A). As water temperature decreases downstream the outflow channels, additional types of cyanobacteria started to appear, as shown in Figs 3B and 3C. Occasionally tightly wound, thin *Spirulina*-like filaments were encountered (Oren et al. 2008). Thus far phylogenetic analyses of environmental samples (see below) did not yield any *Spirulina*-like 16S rRNA gene sequences; however, some of our clones clustered with *Limnothrix*, a genus that includes a (non-thermophilic) spiral organism (*Limnothrix chlorospira*).

The area of the outflow channels in Area B above the waterfalls (Fig. 2D) had mats coloured in part orange, and here we mainly found small *Gloeocapsa*-like unicellular cyanobacteria. More extensive illustrations of the morphological types of cyanobacteria found in the Zerka Ma'in spring area were published elsewhere (Ionescu et al. 2009).

Of special interest is the profuse growth of masses of the branching heterocystous cyanobacterium *Scytonema* observed along some of the outflow channels in the spring area above the waterfalls. The *Scytonema* colonies consist of blackish to dark-green material attached to the rocks (Fig. 4). The colonies are not in direct contact with the hot spring waters, but they are continuously sprayed by small droplets of water from the stream.

We succeeded in growing *Chroogloeocystis* and *Mastigocladus*/*Fischerella* types from samples collected at different places of the Zerka Ma'in thermal area. Some of the iso-

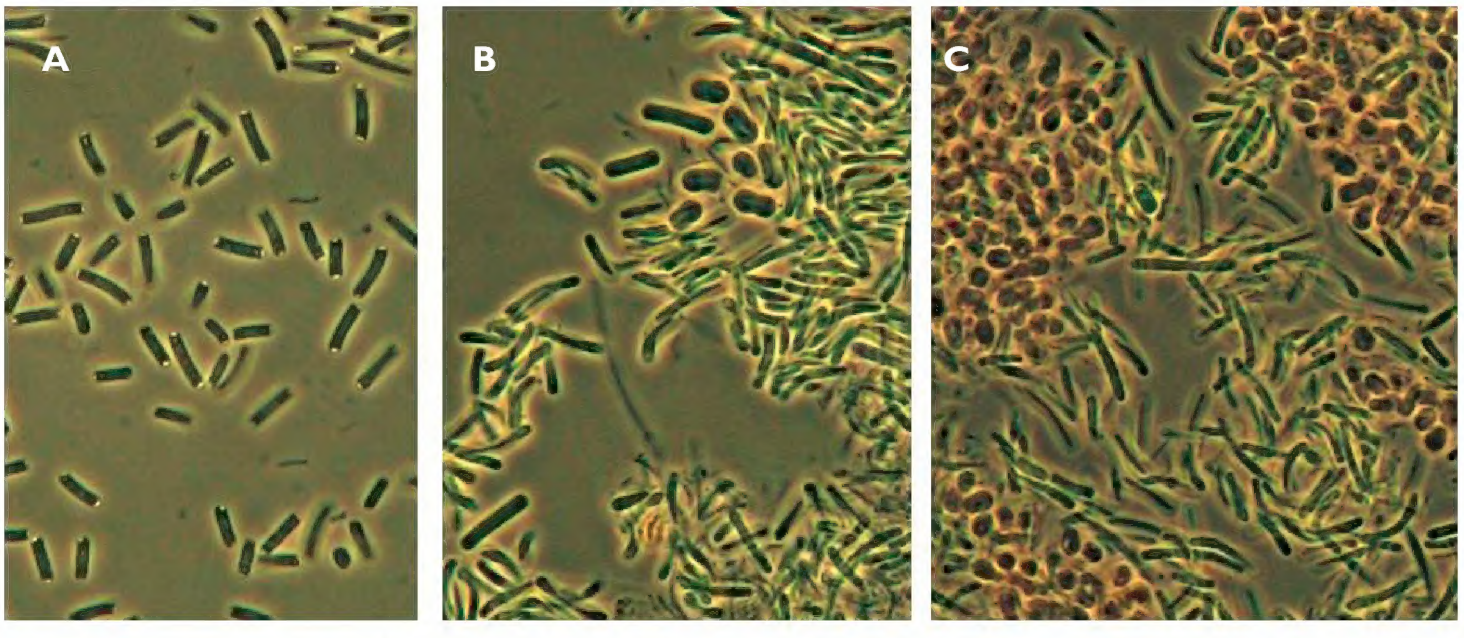

Figure 3. Microphotographs of unicellular cyanobacteria from the Zerka Ma'in hot springs. Different types of unicellular cyanobacteria are shown, collected from the Zerka Ma'in hot springs in November 2006 at temperatures between 58 °C and 63 °C.

lated strains resembled morphologies seen in the field-collected material; others were of types not observed by direct examination of the samples (Ionescu et al. 2007, 2009). The cultures are referred to by accession numbers that start with tBTRCCn (see also Fig. 5). Unfortunately we did not yet succeed in obtaining *Thermosynechococcus*-like organisms from the Zerka Ma'in springs in culture.

16S rRNA gene-based phylogenetic diversity of cyanobacteria

Figure 5 presents the phylogenetic relationships of selected environmental 16S rRNA gene sequences obtained from the Zerka Ma'in springs, indicating the temperature from which the different sequences were recovered, and the sequences of the cyanobacteria grown from the springs as indicated by their tBTRCCn numbers. Only part of the sequences obtained is shown, and similar related sequences are clustered together. For example, the upper box in Fig. 5 ("*Thermosynechococcus*-like clones") is based on 46 distinct and different sequences amplified from the environmental DNA. The *Thermosynechococcus* cluster appears to be particularly diverse in the springs (Oren et al. 2008; Ionescu et al. 2009). More extensive phylogenetic trees were given in Oren et al. (2008).

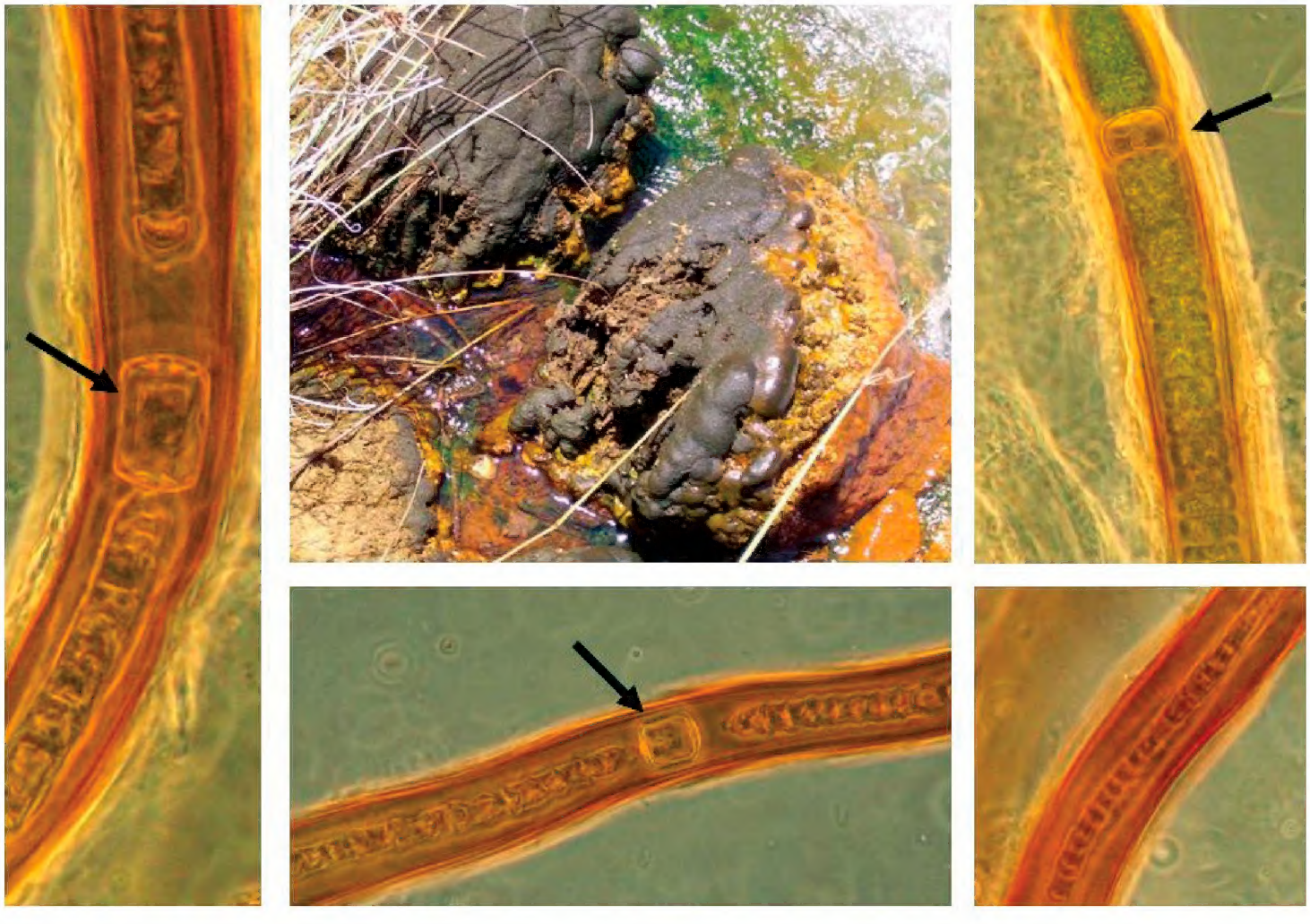

Figure 4. Growth of *Scytonema* in the Zerka Ma'in area. The picture shows growth of large colonies of *Scytonema* on rocks sprayed by water from a thermal stream, and microphotographs of *Scytonema* filaments showing the thick sheath and heterocysts (arrows).

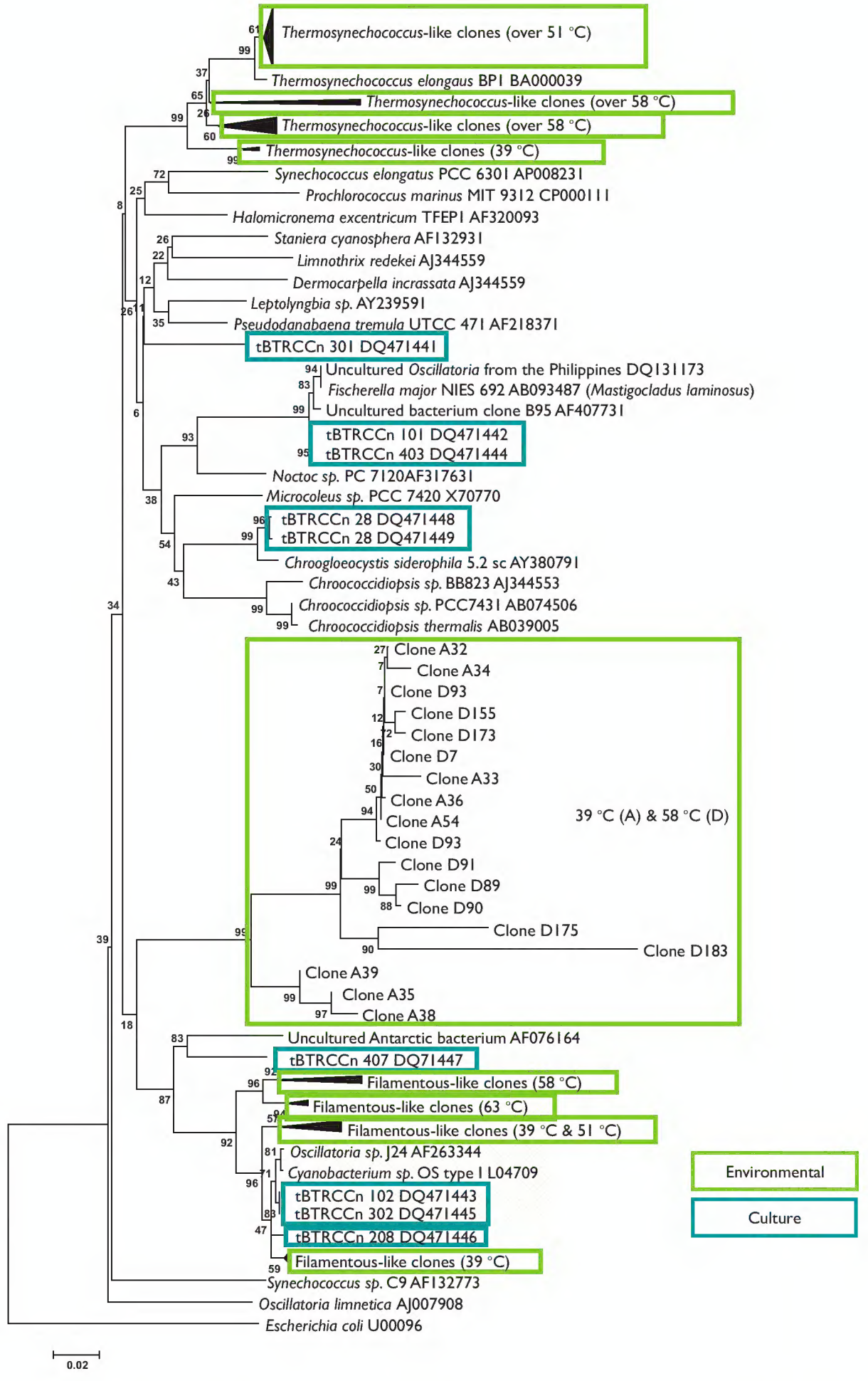

Figure 5. Phylogenetic tree of Zerka Ma'in cyanobacteria, based on 16S rRNA gene sequences. The minimum evolution phylogenetic tree is based on 16S rRNA gene sequences of cyanobacterial isolates obtained from the Zerka Ma'in springs (indicated by tBTRCCn numbers) and on cyanobacterial sequences recovered by PCR amplification from DNA extracted from biomass collected between December 2005 and June 2007 from the thermal springs and outflow channels. The temperature of the waters from which the 16S rRNA genes were recovered is indicated.

Nitrogen fixation studies on the thermophilic cyanobacteria

Chemical analyses of the Zerka Ma'in spring waters (Abu Ajamieh 1980, 1989) show that the concentrations of nitrogen compounds (ammonium, nitrate) are negligible. Therefore the ability to fix gaseous nitrogen will be advantageous to the cyanobacteria that colonise the springs. The observation of heterocysts in the *Scytonema* colonies bordering some of the outflow channels (Fig. 4) suggests that nitrogen fixation by the local cyanobacterial communities may indeed occur.

We detected significant rates of acetylene reduction at all sites sampled, including the 63 °C site. The highest calculated nitrogenase activity (0.0025–0.012 nmol N μg chlorophyll^{-1} h^{-1}) was obtained at 51 °C in the dark. At 63 °C we measured rates of 0.001–0.0025 nmol N μg chlorophyll^{-1} h^{-1}, rates that are low, but significantly higher than background values. Acetylene reduction rates in the light were 30% to 50% lower than those measured in the dark.

The fact that light inhibited nitrogenase activity suggests that the activity is probably located in oxygenic phototrophs, the nitrogenase of which is inhibited by photosynthetically produced oxygen. To test whether mRNA transcripts of the *nifH* (nitrogenase reductase) gene may be present in the Zerka Ma'in cyanobacterial community at the highest *in situ* temperature of 63 °C, we fixed samples in liquid nitrogen; prepared cDNA from the RNA isolated from the community, and amplified gene sequences using *nifH*-specific PCR primers from this cDNA. Using this procedure we isolated a gene identical to a *nifH* gene found in a filamentous cyanobacterial culture obtained from the site. A full account of these experiments was given by Ionescu et al. (2009).

Gene sequences of cyanobacterial *nifH* genes were recovered both from the community DNA and from selected isolates obtained from the spring. Sequences of *nifH* obtained from the environmental DNA were related to those from *Fischerella*, *Phormidium*, and *Lyngbya* spp. (Ionescu et al. 2009). We also sequenced the *nifH* gene of a heterocystous isolate related to *Mastigocladus*/*Fischerella* (strain nBTRCC 101).

Nitrogen fixation by this isolate is now being investigated in further depth. Optimal rates of acetylene reduction were measured at 45 °C (up to 24.5 nmol N μg chlorophyll^{-1} day^{-1}). The maximum temperature for nitrogen fixation in this strain was found to be 52 °C to 53 °C. When grown under light/dark cycles, acetylene reduction rates were higher than under constant light. When a culture grown in nitrate-rich medium was transferred to nitrogen-depleted medium, formation of heterocysts was induced, and acetylene reduction activity started after 48 hours. Quantitative PCR analysis showed expression of the *nifH* gene to be subject to a circadian rhythm. The nature of the phenomenon is currently under investigation.

Discussion

At the highest temperatures (up to 63 °C), unicellular cyanobacteria dominated in the Zerka Ma'in springs area. As water temperature decreases downstream the outflow channels, additional types of cyanobacteria started to appear, including filamentous cyanobacteria belonging to the *Mastigocladus-Fischerella* group, known from thermal springs worldwide (Brock 1978; Castenholz 1969, 1973; Ward and Castenholz 2000). *Spirulina labyrinthiformis* was earlier reported as the dominant organism in material collected by A. Aaronson from a 52 °C spring of Zerka Ma'in (Rayss 1944). Aaronson had joined Blanckenhorn during his above-mentioned 1908 survey of the area (Blanckenhorn 1912), but no further information is available on the exact site and date of collection and no further details have been reported.

The profuse growth of masses of the branching heterocystous cyanobacterium *Scytonema* observed along some of the outflow channels in the spring area above the waterfalls (Fig. 4) is of special interest. It is well possible that these are the "felt-like mats .... in the form of a sponge or pillow with a dark-green, somewhat wrinkled skin", to which Blanckenhorn referred in the quotation given above. Growth of *Scytonema* was also reported from the nearby hot springs of Zara that were surveyed for cyanobacteria and microalgae in 1936 (Frémy and Rayss 1938). The filaments of *Scytonema* are surrounded by a thick, dark brown, layered sheath that has a high content of scytonemin, a dimeric indole alkaloid synthesised from aromatic amino acid residues, which absorbs UV-A radiation (Castenholz and Garcia-Pichel 2000). Qualitative and quantitative information about the content of sctyonemin and other UV-absorbing pigments in the material from Zerka Ma'in has been provided elsewhere (Oren et al. 2008). Scytonemin has its absorbance maximum at 384 nm, with a broad absorption band. Thus the cells are effectively protected against UV-induced cell damage. It remains to be determined, to what extent this property is of importance to the physiology of *Scytonema* at the Zerka Ma'in site. At its location at about 250 m below mean sea level the local level of UV radiation is lower than at higher altitudes, and hardly any traces of other UV-absorbing compounds such as mycosporine-like amino acids could be detected in any other types of cyanobacteria found so abundantly in and around the springs (Oren et al. 2008). Literature data also suggest that the scytonemin content of cyanobacteria that produce the compound may be regulated by factors not directly connected with the light intensity and light quality found in their environment (Castenholz and Garcia-Pichel 2000). It should be noted that *Scytonema* is not a thermophile, and at Zerka Ma'in its colonies are exposed to ambient air temperatures rather than to the temperatures of the thermal spring water (Fig. 4). Surveys of springs in Yellowstone National Park, USA (where UV levels are high at an elevation of > 2000 m above sea level) showed 55 °C to be the upper limit for growth of sheathed, scytonemin-containing species of cyanobacteria such as *Pleurocapsa* and *Calothrix* (Wickstrom and Castenholz 1978).

The isolation of a unicellular cyanobacterium with a 16S rRNA gene with 99% similarity with *Chroogloeocystis siderophila*, an organism originally found in iron-rich

thermal environments in Yellowstone and requiring high iron concentrations for growth (Brown et al. 2005), is remarkable. The Zerka Ma'in waters do not have a high iron content.

Based on the information presented in the tree shown in Fig. 5, a number of interesting conclusions can be drawn: (1) All sequences recovered from the cyanobacteria of Zerka Ma'in appear to be unique, and none of the sequences found was identical to any sequence found in the GenBank database. (2) Some of the Zerka Ma'in organisms have close relatives in other thermal springs worldwide, but there are other types as well that have not been reported from elsewhere. (3) None of the sequences found in our cultures were retrieved directly from the environmental DNA as well. This holds also true for the two cultures of heterocystous cyanobacteria affiliated with the genera *Fischerella* and *Mastigocladus* we have obtained and studied for their nitrogen fixation properties (see below). No related sequences were yet detected among the environmental 16S rRNA gene fragments cloned from the DNA isolated from the site. (4) In many cases sequences found in the lower temperature waters show phylotypes distinct from those present at the higher temperature sites. (5) The sequences in the large box (A32 to A38), which appear to have no equivalent elsewhere, are of special interest. We have no cultured representative of this group yet, so no information is available about the morphology of the organisms that harbor these sequences. Sequences belonging to this group have been retrieved both from 58 °C thermal waters and from a cooler site where we measured 39 °C.

Chemical analyses of the Zerka Ma'in spring waters (Abu Ajamieh 1980, 1989) show that the concentrations of nitrogen compounds (ammonium, nitrate) are negligible. Therefore studies of the nitrogen fixation potential of the cyanobacterial community in the springs were initiated. The finding of nitrogenase at 63 °C was somewhat surprising, as a temperature of 55 °C was generally considered to be the upper limit of nitrogen fixation by cyanobacteria (*Mastigocladus*) in hot spring environments (Fogg 1952, Stewart 1970, Wickstrom 1980). However, the recent finding of transcripts of *nif* genes derived from *Synechococcus* ecotypes in Octopus Spring, Yellowstone National Park, USA, at temperatures up to 63.4 °C (Steunou et al. 2006) suggests that the upper temperature limit of cyanobacterial nitrogen fixation may be higher than previously assumed.

It is intriguing that the unique environment of the Zerka Ma'in hot springs has not been surveyed before by biologists. To our knowledge this is the only site in the Middle East where thermal waters of such high temperatures flow undisturbed and enable the development of a diverse community of phototrophic and other microorganisms adapted to life at temperatures up to 63 °C. The hot springs of Tiberias, Israel, used as a thermal spa since Roman times, reach temperatures very similar to those of Zerka Ma'in. Some exploration of the cyanobacteria present at the site has been done in the past (Dor 1967). However, these springs do not currently flow freely outdoors, so that thermophilic cyanobacteria and other microorganisms adapted to life at high temperatures have little opportunity to develop.

Conclusions

The thermal springs of Zerka Ma'in, Jordan, are inhabited by a great diversity of thermophilic unicellular and filamentous cyanobacteria including *Thermosynechococcus*, *Chroogloeocystis*, *Fischerella* (*Mastigocladus*), and *Scytonema* (occurring as large masses at lower temperatures). Based on 16S rRNA gene sequence analysis, the Zerka Ma'in strains were phylogenetically distinct from morphologically similar cyanobacteria found in hot springs world-wide. Low rates of nitrogen fixation were detected up to 63 °C, the highest temperature recorded in the springs.

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